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Biochimica et Biophysica Acta 1588 (2002) 189–194

BIOCHIMICA ET BIOPHYSICA ACTA

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Correction of the copper transport defect of Menkes patient fibroblasts by expression of two forms of the sheep Wilson ATPase

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Received 14 March 2002; received in revised form 2 July 2002; accepted 25 July 2002

Abstract

The Wilson disease (WD) protein (ATP7B) is a copper-transporting P-type ATPase that is responsible for the efflux of hepatic copper into the bile, a process that is essential for copper homeostasis in mammals. Compared with other mammals, sheep have a variant copper phenotype and do not efficiently excrete copper via the bile, often resulting in excessive copper accumulation in the liver. To investigate the function of sheep ATP7B and its potential role in the copper-accumulation phenotype, cDNAs encoding the two forms of ovine ATP7B were transfected into immortalised fibroblast cell lines derived from a Menkes disease patient and a normal control. Both forms of ATP7B were able to correct the copper-retention phenotype of the Menkes cell line, demonstrating each to be functional copper-transporting molecules and suggesting that the accumulation of copper in the sheep liver is not due to a defect in the copper transport function of either form of sATP7B.

Keywords: Copper metabolism; P-type ATPase; ATP7B

1. Introduction

Copper homeostasis is important because copper is an essential trace element but is toxic in excess, so organisms require mechanisms to maintain intracellular copper at the required level [1]. In humans and other mammals, the liver is the major organ involved in the regulation of overall copper status, with excess copper being excreted from the body in the bile [2]. The importance of the biliary excretion pathway is illustrated by the human copper toxicosis disorder Wilson disease (WD). Patients with this disease cannot excrete copper into the bile, resulting in hepatic copper accumulation and if untreated, fatal copper toxicosis. A second feature of most, but not all WD patients, is very low serum ceruloplasmin levels [3]. The gene that is defective in WD patients encodes a copper-transporting P-type ATPase (ATP7B) [4–6]. ATP7B has two functional roles in copper homeostasis in the liver. The first is the transport of copper into the lumen of the Golgi apparatus for incorporation into secreted copper-dependent enzymes, primarily ceruloplasmin [7]. The second role of ATP7B is the excretion of excess copper into the bile. This involves copper-induced trafficking of ATP7B from the trans-Golgi network (TGN) or late endosomic vesicles, to vesicles and the apical biliary canalicular membrane when copper is present in excess [8–10]. The dual roles of ATP7B may explain the heterogeneity in clinical features of patients presenting with WD, with different ATP7B alleles affecting either copper transport or intracellular location/trafficking of ATP7B [8]. Recently, a cell culture system was utilised to distinguish ATP7B variants in which copper transport was disrupted from those in which intracellular trafficking was impaired [11].

Sheep have a variant copper phenotype when compared with other mammals. The species is prone to copper deficiency due to the formation of copper-chelating thiomolybdates in the rumen [12]. However, sheep are prone to copper toxicosis due to a reduced capacity to excrete copper in the bile. Unlike most mammals, the rate of biliary copper excretion is not responsive to copper intake [13]. Analogous

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0925-4439/02/$ - see front matter
PII: S0925-4439(02)00164-3

to WD patients, the intake of moderate dietary levels of copper can result in elevated hepatic copper concentrations, liver failure and death [14]. Unlike most WD patients, the level of copper and holoceruloplasmin in sheep plasma is similar to the normal human range [15]. We are investigating the molecular basis of the sheep copper phenotype. In previous studies, we cloned the sheep *ATP7B* (*sATP7B*) cDNA, but did not find any obvious abnormality of structure or expression that would suggest that sheep have a defect in ATP7B. However, we identified a novel form of the *ATP7B* cDNA which had an alternative N-terminal region, generated by alternate splicing [16]. The form corresponding to published ATP7B orthologues (normal sATP7B) encodes an 18-amino-acid N-terminal sequence, which is replaced by a novel 79-amino-acid sequence in the alternate sATP7B protein. Both forms of sATP7B localise to the TGN and undergo copper-induced trafficking to a vesicular compartment when expressed in CHO cells [17].

In this study, we examined the copper-translocating activity of each form of sATP7B to determine if the sheep copper phenotype might be related to alterations in this activity in either form of sATP7B. The copper transport function of both forms of sATP7B was examined in vitro by the generation of fibroblast cell lines from a Menkes disease patient and a normal control, which expressed cDNA-derived sATP7B. Although exogenously expressed ATP7B does not undergo copper-induced trafficking in this cell line, the copper transport function of introduced ATPases can be accurately assessed by measuring efflux of radiolabeled copper from the cell. Our results indicate that both forms of sATP7B correct the copper accumulation defect of Menkes patient fibroblasts and therefore are functional copper-transport proteins.

2. Materials and methods

2.1. Cell culture, constructs and antibodies

The generation and characterisation of immortalised fibroblasts derived from a Menkes disease patient (Me32a-T22/2L) and control cell line (GM847) has been previously described. The Me32a MNK gene has a 4-base-pair (bp) deletion within exon 6, resulting in a prematurely terminated protein between metal binding sites 5 and 6, and complete loss of the protein as determined by Western blot utilising a polyclonal antibody directed against all six metal binding sites [18]. Cells were maintained at 37 °C in basal medium (approximately 1 μM copper), which consisted of Eagle's basal medium (BME) (Trace BioSciences) supplemented with 2 mM L-glutamine, 0.2 mM proline, 1.2 mM $NaHCO_3$, 20 mM HEPES and 10% foetal calf serum (Commonwealth Serum Laboratories). The generation of the constructs pNORM and pALT, encoding the normal and alternate forms of sheep ATP7B cloned into a low-copy number mammalian expression vector was reported previously, as was the production, purification and characterisation of antibodies specific for each form of sheep ATP7B [17].

2.2. Cell transfection and expression analysis

The transfection of cells was performed essentially as described previously [17]. Briefly, stable transfection of cultured cells was performed with 20 μg of linear plasmid DNA and Superfect reagent (Qiagen) according to the manufacturer's protocols. Following 3 weeks of G418 selection (Gibco, 400 μg/ml), resistant colonies were detached, expanded and maintained as a mixed population of G418-resistant cells. Approximately 60–80% of the G418-resistant population expressed sATP7B, as determined by immunofluorescence analysis (see below). The transfected cell populations were routinely cultured in the presence of G418 (200 μg/ml) to maintain selective pressure for expression of the ATP7B transgene. The generation and characterisation of Me32a-T22/2L cells expressing mouse Atp7b was reported previously [18]. Total RNA was prepared from cells and the expression of each ATP7B transcript was analysed utilising a ribonuclease protection assay (RPA) as described previously [16]. Total protein extracts were prepared from approximately 5×10^6 cells as previously described [19]. The protein concentration was estimated by absorbance at 280 nm and approximately 15 μg of each sample was separated by SDS-PAGE on 7.5% gels (Bio-Rad) under reducing conditions and transferred to nitrocellulose membranes (Millipore). Immunoblot analysis was carried out using the BM Chemiluminescence Kit (Boehringer Mannheim) according to the manufacturer's protocols. Filters were incubated overnight at 4 °C in blocking buffer and incubated for 1–5 h with the appropriate affinity-purified primary antibody at room temperature. After four washes in blocking solution, filters were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-rabbit IgG (Amrad Pharmacia Biotech) and immunoreactive proteins were visualised by chemiluminescence.

2.3. Immunofluorescence microscopy

Immunofluorescence analysis of cells was carried out essentially as described previously [20]. Where appropriate, copper (as $CuCl_2$) was added to the growth medium. In general, cells were grown to 60% confluence on glass cover slips, fixed with 4% paraformaldehyde in PBS for 5 min, permeabilised with 0.1% Triton X-100 in PBS for 5 min at room temperature and placed in blocking solution (1% BSA/1% gelatin in PBS) overnight at 4 °C. Primary antibodies consisted of affinity-purified anti-normal and anti-alternate sheep ATP7B antibodies (1:100 or 1:50) and an affinity-purified anti-mouse Atp7b (1:50) antibody described previously [18]. The secondary antibody was affinity-purified FITC-conjugated sheep anti-rabbit IgG (Amrad Pharmacia Biotech, 1:400). The cover slips were

mounted onto glass slides using 2.6% DABCO (1,4-diazabicyclo-(2.2.2) octane, Sigma) in 100% glycerol and analysed using a Zeiss axioskop fluorescence microscope with a CCD camera (Photometrics) and V for Windows software (Digital Optics).

2.4. ^{64}Cu accumulation and retention studies

Copper accumulation studies were performed as described [18]. Briefly, cells were cultured in basal medium and transferred to BME supplemented with 2% foetal calf serum and trace amounts of ^{64}Cu (approximately 0.5 μM as $CuCl_2$; Australian Radioisotopes). For accumulation studies, cells were incubated for 24 h at 37 °C, washed twice in cold BME, and harvested by homogenisation in 0.1% SDS. For retention experiments, cells were treated as above but with an 8-h incubation in BME containing 2% foetal calf serum but lacking ^{64}Cu. The cells then were washed twice with cold BME and harvested in 0.1% SDS. The amount of ^{64}Cu in the cell lysates was measured using an LKB-Wallac Ultragamma counter and the protein content of the extracts was determined by protein assay [21].

3. Results

3.1. Transfection and expression analysis of sheep ATP7B in Menkes patient and control fibroblasts

Expression constructs encoding the normal (pNORM) and alternate (pALT) sATP7B cDNAs were transfected into immortalised fibroblast cell lines, one control, GM847 and one derived from a Menkes disease patient, Me32a-T22/2L.

To confirm expression of the sheep *ATP7B* cDNA, total RNA was extracted from 5×10^6 cells and 3 μg was analysed by a RPA. A protected fragment of the expected size (approximately 180 bp) was observed in Me32a-T22/2L and GM847 cells transfected with pNORM, but not in the corresponding untransfected cells or those transfected with pALT (Fig. 1). Similarly, a protected fragment of expected size (approximately 340 bp) was detected in cells transfected with pALT but not in untransfected cells or pNORM-transfected cells (Fig. 1).

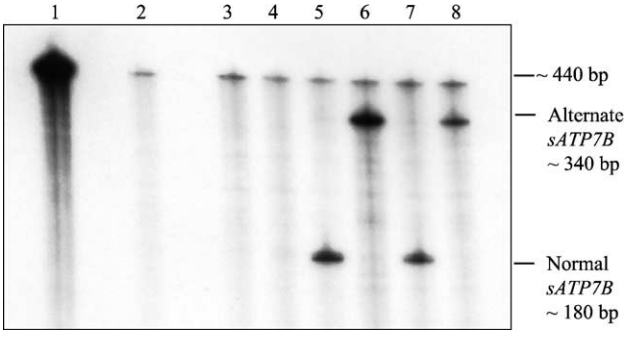

Fig. 1. RPA of *sATP7B* expression in transfected fibroblast cells. Total RNA was isolated from transfected cells and 3 μg was analysed by RPA as described in the Materials and methods. Lane 1 shows the size of the full-length, undigested probe and lane 2 the probe treated with ribonuclease. The RNA samples are: lane 3, untransfected Me32a Menkes patient fibroblasts; lane 4, untransfected GM847 normal fibroblasts; lane 5, Me32a transfected with pNORM; lane 6, Me32a transfected with pALT; lane 7, GM847 transfected with pNORM; lane 8, GM847 transfected with pALT. The approximately 340 and 180 bp protected fragments correspond to the alternative and normal *sATP7B* transcripts, respectively.

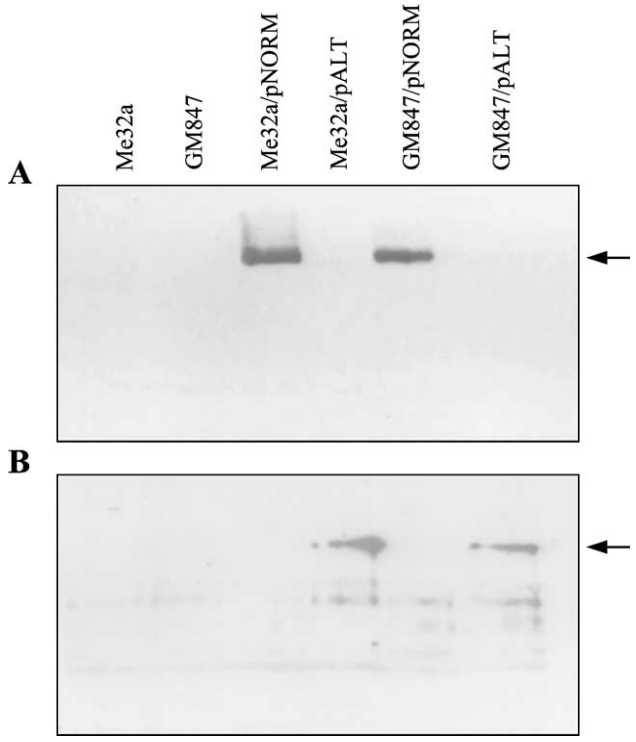

Fig. 2. Western blot analysis of sATP7B expressed in fibroblast cells. Approximately 15 μg of total protein extract from fibroblast cells was fractionated by SDS-PAGE (7.5%) and duplicate gels were transferred to nitrocellulose membranes. Sheep ATP7B was detected with affinity-purified anti-normal sATP7B (A, 1:100) or anti-alternate sATP7B antibody (B, 1:50) and enhanced chemiluminescence. The samples are: Me32a, untransfected Me32a Menkes patient fibroblasts; GM847, untransfected GM847 normal fibroblasts; Me32a/pNORM, Me32a transfected with pNORM; Me32a/pALT, Me32a transfected with pALT; GM847/pNORM, GM847 transfected with pNORM; GM847/pALT, GM847 transfected with pALT. The position of the approximately 160 kDa sATP7B protein is indicated.

Western blot analysis was carried out to confirm the expression of sheep ATP7B and to determine the relative level of protein expression. Approximately 15 μg of whole cell protein extract from the transfected and control cell lines was analysed. A protein of the expected size (approximately 180 kDa) was detected in the appropriate cell lines following analysis with either the anti-normal or anti-alternate ATP7B antibody (Fig. 2). There was no evidence of ATP7B protein in the untransfected cell lines. This result was expected as these cells do not express detectable levels of endogenous human ATP7B mRNA [18] and the sheep ATP7B antibodies do not cross-react with human ATP7B or human ATP7A (data not shown). The lower molecular

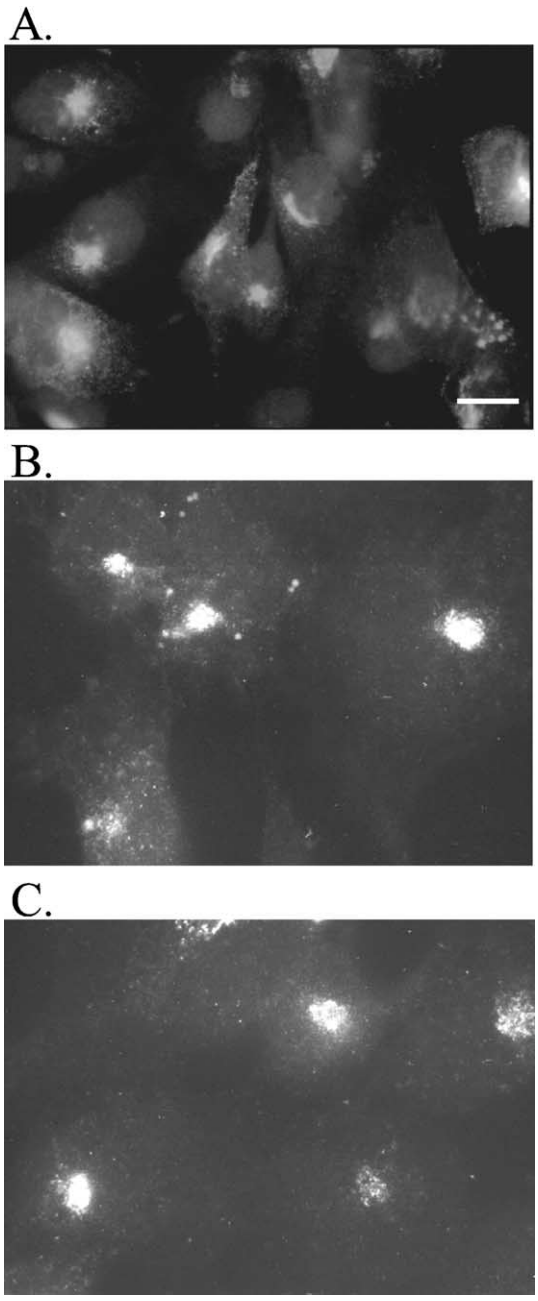

Fig. 3. Immunofluorescence microscopy analysis of ATP7B expressed in fibroblast cells. Transfected Menkes patient fibroblast cells expressing normal sATP7B (A), alternate sATP7B (B) or mouse Atp7b (C) were grown in basal media. The cells were fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100 before staining by indirect immunofluorescence with affinity-purified antibodies raised against normal sATP7B (1:100), alternate sATP7B (1:50) and mouse Atp7b (1:50). Primary antibodies were detected using affinity-purified FITC-conjugated sheep antibodies to rabbit IgG. Bar: 10 μm (× 60 objective).

weight bands observed with the anti-alternate sATP7B antibody probably represent non-specific background, since they also were visible in the untransfected samples following an increased exposure time (data not shown).

3.2. Intracellular distribution of sheep ATP7B in Menkes patient and control fibroblasts

Immunofluorescence analysis was used to confirm expression and assess the intracellular distribution of each form of the sheep ATP7B protein in Menkes patient and control fibroblasts. In basal medium, each form of sheep ATP7B was similarly localised to the perinuclear region of transfected Menkes patient cells (Fig. 3A,B). A similar distribution was observed for each form of sATP7B in the control cell line (data not shown). This localisation of sATP7B was similar to that of murine Atp7b when expressed in the same cell line (Fig. 3C) and was consistent with previous studies, which demonstrated that sheep, mouse and human ATP7B is located at the TGN [17,18,22].

3.3. Copper accumulation and retention studies in transfected fibroblasts

Copper transport studies were carried out to determine if the sheep ATP7B proteins could correct the abnormal copper accumulation of Menkes patient fibroblasts, as was reported for mouse and human ATP7B [18,23]. Following a 24-h incubation in media containing approximately 0.5 μM ^{64}Cu, the parental Me32a cell line accumulated approximately three-fold more copper than the control GM847 cell line (Table 1). The copper accumulation in Me32a cells transfected with either form of sheep ATP7B was significantly reduced in comparison to the non-transfected Me32a cells and was comparable to, or lower than the copper accumulation of the control cell line GM847 (Table 1). Further, the copper accumulation of normal fibroblasts (GM847) transfected with either form of sheep ATP7B

Table 1
Copper accumulation and efflux by fibroblasts expressing sATP7B

Cell line	24 h Copper accumulation (pmol Cu/μg protein, mean ± S.D.)	Percentage copper retention (mean ± S.D.)
Me32a	0.65 ± 0.04	76 ± 1.2
GM847	0.25 ± 0.01	49 ± 1.7
Me32a–pNORM	0.2 ± 0.03	36 ± 1.5
Me32a–pALT	0.006 ± 0.001	30 ± 2
GM847–pNORM	0.006 ± 0.001	34 ± 3.5
GM847–pALT	0.006 ± 0.001	32 ± 1.7

Transfected and untransfected Menkes patient fibroblast (Me32a) and control fibroblast cells (GM847) were grown in ^{64}Cu-containing media for 24 h and the amount of copper accumulated in the cells was determined. The percentage copper retention represents the amount of copper retained in the cells after a further 8 h incubation in media lacking ^{64}Cu, expressed as a percentage of the amount of ^{64}Cu accumulated in cells during the 24-h period. The samples are: Me32a, untransfected Menkes patient fibroblasts; GM847, untransfected normal fibroblasts; Me32a–pNORM, Me32a transfected with pNORM; Me32a–pALT, Me32a transfected with pALT; GM847–pNORM, GM847 transfected with pNORM; GM847–pALT, GM847 transfected with pALT. Values represent the mean ± S.D. of three independent measurements.

was significantly reduced in comparison to the non-transfected GM847 cells. After a further 8-h incubation in medium without ^{64}Cu, the amount of radioactive copper retained within the cells was determined, to give an indirect measure of copper efflux. The results demonstrated that Menkes or normal cells transfected with either form of sheep ATP7B retained significantly less copper than the parental cell lines (Table 1), suggesting that sheep ATP7B corrected the copper transport defect in these cells by enhancing copper efflux, rather than by decreasing copper uptake.

4. Discussion

The results of previous studies of the structure and expression of *sATP7B* suggested that the gene could encode a functional protein [16]. Similarly, in vitro analysis of sATP7B demonstrated interaction with the copper chaperone SAH [24] and copper-induced trafficking in Chinese hamster ovary (CHO) cells [17], properties required for correct functioning of ATP7B. However, copper homeostasis mediated by ATP7B is proposed to involve copper transport across cellular membranes in addition to ligand-induced trafficking of the molecule when copper is in excess [8]. In this study, we demonstrated that the two forms of sATP7B are functional copper-transporting molecules.

A diagnostic feature of Menkes disease is that cultured cells derived from an affected individual hyper-accumulate copper [25,26]. The functional complementation of the copper accumulation phenotype of Menkes patient fibroblasts by ATP7A and ATP7B has demonstrated that these cells constitute a valuable model for analysis of the copper transport function of CPx-type ATPases [18,23]. RNA and protein analysis confirmed the successful transfection and expression of cDNA-derived sATP7B in Menkes and control fibroblasts. A significant level of sATP7B mRNA and protein was detected in transfected fibroblasts, but not in untransfected cells (Figs. 1 and 2).

Immunofluorescence analysis was used to assess the intracellular distribution of sATP7B in the Menkes and control fibroblasts and demonstrated that the two proteins were similarly localised within the cell. In basal medium, each form of sATP7B was localised to the perinuclear region in transfected Menkes patient cells (Fig. 3). This localisation was observed in cells expressing different levels of the recombinant protein and was consistent with previous studies which indicated that ATP7B is located at the TGN in basal copper conditions [17,22]. The addition of 200 μM copper to the medium did not appear to significantly alter the localisation of sATP7B in the transfected cells (data not shown). A similar apparent lack of response to copper was observed when the cDNA-derived murine Atp7b protein was expressed in the Me32a and GM847 cell lines [18], suggesting that normal trafficking of ATP7B does not take place in these fibroblast cells. In contrast, a significant proportion of human ATP7B expressed in *mottled* mice fibroblasts and HepG2 cells underwent a copper-induced relocalisation to a vesicular, cytoplasmic compartment [22,23]. It is difficult to reconcile these conflicting results, but they do suggest that the cell type utilised for in vitro studies may significantly influence the extent to which copper-induced relocalisation of ATP7B is detectable.

Copper transport studies demonstrated that both forms of sATP7B corrected the copper accumulation of Menkes patient fibroblasts. Following a 24-h incubation in medium containing trace amounts of ^{64}Cu, the parental Me32a cell line accumulated approximately three-fold more copper than the control GM847 cell line (Table 1). The copper accumulation in Me32a cells transfected with either form of sATP7B was reduced in comparison to the non-transfected Me32a cells and was comparable to the copper accumulation of the control GM847 cell line. The reduced copper accumulation of the Me32a cells transfected with pALT, in comparison to pNORM, might have been due to higher expression levels of alternate sATP7B, as suggested by the intensity of the protected fragment in the RPA analysis (Fig. 1). Alternatively, the difference may be the result of a larger proportion of the G418-resistant population expressing pALT in comparison to pNORM. Similarly, the copper accumulation of normal fibroblasts (GM847) transfected with either form of sATP7B was significantly reduced in comparison to the non-transfected GM847 cells. These observations were analogous to those reported for functional murine and human ATP7B expressed in Menkes and mottled fibroblasts [18,23]. After a further 8 h incubation in medium without ^{64}Cu, the amount of radioactive copper retained within the cells was determined, to give an indirect measure of copper efflux. The transfected Me32a and GM847 cells expressing sATP7B retained similar proportions of copper as the non-transfected control cell line, and this was substantially less than the non-transfected Me32a cell line (Table 1). This result suggests that sATP7B corrected the copper transport defect in these cells by enhancing copper efflux, rather than by decreasing copper uptake. These results demonstrate that both forms of sATP7B are functional copper-transport proteins that can reduce the intracellular copper content of fibroblasts by enhancing copper efflux. Taken together with our previous studies examining the structure, expression and localisation of sATP7B [16,17,24], these results suggest that the copper toxicosis syndrome of sheep may not result from defects in the copper transport or localisation of either form of sheep *ATP7B*. It is important to note that these studies utilised a non-polarised cell culture system and the sheep ATP7B proteins may have properties distinct from other mammalian copper ATPases that are only apparent in hepatic cells which have an apical (canalicular) and basolateral surface. Furthermore, the specific identity of the compartment to which ATP7B traffics in response to elevated copper is yet to be determined. If this is found to be different for the two forms of sATP7B, it may suggest distinct roles for these proteins in copper homeostasis. Future studies will be directed toward

comparison of the sheep proteins in a polarised hepatic cell line.

Acknowledgements

We thank Rosario Reyes for invaluable technical support and express our sincere appreciation for the advice and support given by our laboratory colleagues. This work was supported in part by the National Health and Medical Research Council of Australia (NH and MRC), the Australian Research Council and the International Copper Association. S.L. is supported by an NH&MRC R. Douglas Wright Award.

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